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Solubilization in high yield of opioid receptors retaining high-affinity delta, mu and kappa binding sites

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The binding sites for opiates (agonist and antagonist) and opioid peptides can be solubilized from rat brain membranes with digitonin in the presence of Mg^{2+} (10 mM). High affinity and high capacity binding to the soluble δ, μ, and $\varkappa$ receptors is obtainable when the membranes are treated in Mg^{2+} (30°C, 60 min) prior to solubilization. The yields of solubilized binding sites extracted with digitonin, 40–90%, are higher than those obtained from Mg^{2+}-pretreated membranes with other detergents commonly used for receptor solubilization. The stability of the digitonin-soluble opioid receptor at room temperature makes it useful for purification and characterization.

Opioid receptor *Receptor solubilization* *Subtype*

1. INTRODUCTION

While it is well established from studies in situ and at the membrane level that the opioid receptor exists in multiple subtypes, δ, μ, $\varkappa$ [1] and perhaps others, the molecular basis for this heterogeneity has not been examined in the isolated state, due to the loss of all or most of the opioid peptide binding in detergent solutions. Relatively low yields of the δ-receptor activity have been obtained from rat brain and from neuroblastoma × glioma cell membranes using the Zwitterionic detergent CHAPS [3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulphonate] [2,3]. Likewise, rat brain yielded CHAPS-soluble activity for $\varkappa$ and μ ligands but of much lower specific activity than that present in the starting membranes [3,4]. Opioid binding activity has been reported in 1% Triton X-100 extracts of rat brain membranes [5], but this required removal of the detergent by adsorption and the yield is unknown. Opioid activities have been solubilized from toad brain [6] and mammalian brain using the nonionic detergent digitonin plus 1 M NaCl [7]. However, this activity was labile above 4°C and in these preparations peptide binding at δ and μ sites was not seen.

In our work we have taken an alternative approach which also employs digitonin, but takes advantage of the stabilization of receptor activity by interaction with Mg^{2+} in the membranes. Enrichment of soluble, high-affinity δ, μ and $\varkappa$ receptor sites and also opiate antagonist binding sites is achieved thus. The soluble activity is then stable at 30°C. Comparisons are made here with the soluble receptor activity obtainable with other detergents.

2. MATERIALS AND METHODS

2.1. *Materials*

[15,16-^{3}H]Etorphine (27–45 Ci/mmol), D-Ala^2-[*tyrosyl*-3,5-^{3}H]-D-leu^5-enkephalin (DADLE) (15–40 Ci/mmol), [*tyrosyl*-3,5(n)^{3}H]dynorphin(1–9) (20–50 Ci/mmol), [^{3}H]dihydromorphine (65–90 Ci/mmol), [*N-allyl*-2,3-^{3}H]naloxone (40–60 Ci/mmol) were from Amersham. Triton X-100, Lubrol-PX, polyethylene glycol (PEG, type P-2139), gamma-globulin, *N*-tris [hydroxymethyl]methyl-2-aminoethanesulphonic acid (Tes), Tris, BSA, soybean trypsin inhibitor, bacitracin and benzamidine were from Sigma. CHAPS and cholic acid were from Serva. Na^+-deoxycholate was from Koch-

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Light and digitonin from BDH. (±)-Bremazocine was a gift from Dr R. Maurer, Sandoz.

2.2. *Membrane preparation and solubilization procedures*

Brain membranes were prepared [8] from rat brain (minus cerebellum) and stored at −70°C, at a concentration of 10–12 mg/ml. They were thawed and resuspended at 0.5 mg protein per ml in ice-cold Tes–KOH buffer (10 mM, pH 7.5) containing EGTA-K^+ (1 mM), $MgSO_4$ (10 mM), benzamidine–HCl (1 mM), bacitracin (0.01%) and soybean trypsin inhibitor (0.002%), referred to as 'Mg buffer'. The membranes were pelleted at 40000 × *g* (4°C, 30 min), resuspended in Mg buffer at 2–3 mg/ml, incubated at 30°C for 60 min, diluted 1:4 with Mg buffer and pelleted as above.

Digitonin was dissolved (10%, w/v) in Tes–KOH buffer (10 mM, pH 7.5) at 100°C; after cooling at 5°C, the small amount of digitonin precipitated was removed by filtration. Preincubated membranes were resuspended in Mg buffer containing digitonin (2%, w/v) at 2:1 detergent:protein. The membranes were shaken gently for 60 min at room temperature and the 120000 × *g* (5°C, 60 min) supernatant was collected.

For other detergents, membranes preincubated in Mg buffer as above were solubilized in the same buffer, at 5–6 mg protein/ml containing the detergent as stated, by gently shaking at 5°C for 30–60 min and centrifugation as above.

2.3. *Binding activity*

The detergent finally present in the assay was 0.1%, or 1 mM for CHAPS. The assay medium (final volume 0.5 ml) contained in Mg buffer the radiolabelled ligand at the stated concentration and the receptor at a final protein concentration of 0.06–0.17 mg/ml. Non-specific binding was measured in the presence of 1–2 × 10^{-6} M unlabelled ligand. The samples were incubated at 30°C for 2 h, then cooled on ice for 5 min. At 0°C, gamma-globulin (50 μl of 0.6% in 10 mM Tes–KOH, pH 7.5) was added to each sample, followed by 200 μl PEG (36% in 10 mM Tes–KOH, pH 7.5), with incubation for 30 min. Aliquots (300 μl or 600 μl) were filtered (Whatman GF/C filters) under suction, washed rapidly with 4–5 ml ice-cold PEG (10% in 5 mM Tes–KOH, pH 7.5), dried and counted. Protein was determined by the mini-Lowry method [9] after precipitation with 12% trichloroacetic acid.

3. RESULTS

3.1. *Opioid receptor activity solubilized by different detergents*

Pretreatment of membranes with Mg^{2+} (10 mM) at 30°C for 60 min prior to solubilization with digitonin (2%) resulted in a highly active soluble receptor. High specific binding for [^{3}H]etorphine and [^{3}H]DADLE was retained (table 1). Solubilization with the Zwitterionic detergent CHAPS (10 mM) yielded a receptor preparation which, although it again displayed specific binding for [^{3}H]etorphine and [^{3}H]DADLE, had a lower activity than that obtained in digitonin (table 1). In 1 mM CHAPS in the assay medium, non-specific binding was then 60% of the total (at 12.4 mM ^{3}H-ligand), whereas it was only 30% for digitonin (assayed at 0.1% detergent). This suggests that at concentrations of CHAPS below its critical micellar concentration of 4–5 mM [10] there may be some protein aggregation. Solubilization with the non-ionic detergents Lubrol-PX or Triton X-100, or the ionic detergents K^+-cholate or Na^+-deoxycholate, resulted in soluble receptor preparations which did not bind [^{3}H]DADLE (even when the detergent was finally diluted to 0.1%) and retained only a low opiate-binding activity (table 1). A higher activity was obtained for [^{3}H]etorphine in the Na^+-deoxycholate receptor preparation (table 1). This activity was suppressible by 10 nM bremazocine (by >80%, at 3 nM [^{3}H]etorphine), suggesting that this detergent stabilizes mainly $\varkappa$-sites. Since no activity was observed in K^+-cholate, however, these results suggest that it is probably the Na^+ component in the Na^+-deoxycholate which stabilizes the $\varkappa$ receptor activity. This is in agreement with the high $\varkappa$ activity obtained for receptor solubilized with digitonin in the presence of 1 M NaCl [7]. The non-specific binding in these other 4 detergents ranged from 60% to 80% of total ligand bound.

The receptor solubilized in 2% digitonin retained its activity after freezing (in liquid N_2) and storage (up to at least 3 months) at −70°C; the activity was retained after incubation for up to 4 h at room temperature, and has been found stable

Table 1

The effects of various detergents on the solubilization of [^{3}H]etorphine and [^{3}H]DADLE specific binding activity

Detergent	% protein solubilized	% yield	
		[^{3}H]Etorphine	[^{3}H]DADLE
Digitonin (2%)	45	90 ± 4	45 ± 1
CHAPS (10 mM)	22	6 ± 3	8 ± 3
Triton X-100 (1%)	35	1 ± 0.5	[a]
Lubrol-PX (1%)	30	4 ± 2	[a]
Na^+-deoxycholate (1%)	61	52 ± 11	[a]
K^+-cholate (1%)	42	6 ± 4	[a]

[a] No significant differences were obtained here between total and non-specifically bound radiolabelled ligand

Receptor was solubilized from membranes pretreated in Mg buffer (30°C, 60 min) with the detergent concentration shown and assayed at 0.1% detergent (or 1 mM for CHAPS) in the presence of Mg^{2+} (10 mM). Results are the means ± SE of triplicate determinations. For digitonin and CHAPS, 100% activity is the B_{max} value for the membrane-bound receptor obtained under similar ionic conditions. For the other detergents a single high ligand concentration was tested (25 nM for [^{3}H]etorphine; 20 nM for [^{3}H]DADLE) and the activity is compared with that obtained with the membrane-bound receptor at equimolar ligand concentration

enough for further purification. The highest specific activity for [^{3}H]DADLE binding in digitonin was obtained when the concentration of the detergent was diluted to 0.1% [6] in the assay medium (table 2). Non-specific binding at this concentration of detergent was only 30% (at 11 nM [^{3}H]DADLE) of total ligand bound. In 0.1% digitonin/Mg^{2+} solution there was no loss of soluble activity after centrifugation at 100000 × g at 5°C for 30 min.

Table 2

Effect of digitonin concentration on [^{3}H]DADLE binding activity

Detergent concentration (%, w/v)	Specific activity (pmol/mg protein)
2.0	0.001 ± 0.001
1.0	0.051 ± 0.040
0.5	0.054 ± 0.010
0.2	0.106 ± 0.030
0.1	0.210 ± 0.020[a]

[a] Total radioactivity bound was 397 ± 20 cpm/ml and non-specific 123 ± 17 cpm/ml

Specific binding of [^{3}H]DADLE (11.2 nM) was measured in the presence of Mg^{2+} (10 mM) and the detergent concentrations stated, at 0.096 mg protein/ml. Non-specific binding was measured in the presence of unlabelled DADLE (1.63×10^{-6} M). Aliquots filtered were 600 μl. Results are the means ± SE of triplicate determinations

3.2. *PEG precipitation of the receptor*

The amount of ^{3}H-ligand specifically bound to the soluble receptor was dependent on the amount of PEG (10%) used to wash the PEG-precipitated ligand–receptor complex on the filters. For the digitonin solubilized receptor, at a ligand concentration of 5–6 nM, the precipitated activity recovered in the presence of Mg^{2+} (10 mM) was 98% after two washings with 3 ml 10% PEG (100% activity was that obtained after one wash with 3 ml), or 60–70% after 4 × 3 ml washings. The results reported here are those obtained after one wash with 4 or 5 ml PEG, which gives negligible ligand dissociation but adequately low non-specific binding. The free ligand concentrations quoted are on the basis of the 0.5 ml volume used in the incubation, and not of the 0.75 ml final

volume after addition of the gamma-globulin and the PEG (i.e., we assume no re-equilibration, which if it occurs would mean that the receptor affinities are even higher than stated).

3.3. *Binding of ^{3}H-ligands to receptor solubilized with digitonin*

The binding isotherms for [^{3}H]DADLE and for the κ-ligand [11] [^{3}H]dynorphin(1–9), with receptor solubilized in digitonin, are shown in fig.1a. The total number of binding sites (B_{max}) obtained for [^{3}H]DADLE was 0.45 pmol/mg protein, as obtained from a Scatchard plot of the data (not shown). This specific activity was identical to that obtained under equivalent conditions with the membrane-bound receptor. From the Hill plot of these data (fig.1b) K_d = 7.0 ± 0.5 nM (n_H = 1.33 over 10–90% saturation, correlation coefficient (r) = 0.99) was obtained; this compares with K_d = 2.0 nM obtained for [^{3}H]DADLE in the membranes under similar conditions. Forty percent of the total [^{3}H]DADLE binding activity was suppressible by the μ-ligand dihydromorphine at 25 nM, showing that both δ- and μ-receptor sites are present in this receptor preparation. The total yield of [^{3}H]DADLE-binding soluble activity (δ plus μ sites) was 45%.

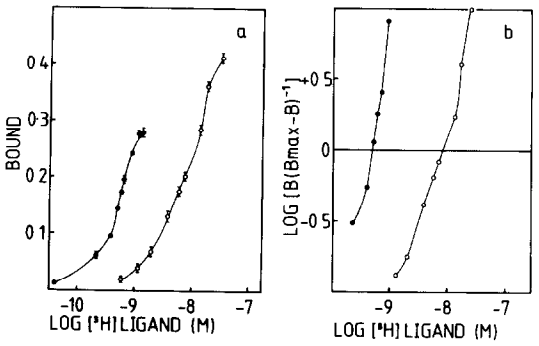

Fig.1. Semi-log (a) and Hill plots (b) for [^{3}H]DADLE (○) and [^{3}H]dynorphin(1–9) (●) binding to digitonin-soluble receptor. Specific binding (B, in pmol/mg protein) was assayed in the presence of Mg^{2+} (10 mM) at 0.1% digitonin. Non-specific binding for [^{3}H]DADLE was measured in the presence of unlabelled DADLE (1.6×10^{-6} M), or for [^{3}H]dynorphin(1–9) in the presence of (±)-bremazocine (2×10^{-6} M). The protein concentration was 0.06–0.09 mg/ml. Results are the means ± SE of 3 independent determinations. Error bars in (a) are shown only when larger than the symbols used.

Table 3

Specific binding activities of digitonin-solubilized receptor

^{3}H-ligand	B_{max} (pmol/mg protein)
Etorphine	1.26
Dihydromorphine	1.26
Naloxone	0.45
DADLE	0.45
Dynorphin(1–9)	0.27

The mean B_{max} was measured from Scatchard plots of data as in fig.1 (for DADLE or dynorphin(1–9)) or is given as the specific binding at 20–25 nM ^{3}H-ligand (shown to be essentially saturating) at 30°C. Non-specific binding (with 10^{-6} M unlabelled ligand present) was <40% of the total binding (60% for etorphine) under these conditions. The SE of the mean was <0.02 pmol/mg in every case

The total number of sites labelled specifically by [^{3}H]dynorphin(1–9) (fig.1a) was 0.27 pmol/mg protein, as obtained from the Scatchard plot of these data (not shown). From the Hill plot (fig.1b) K_d = 0.4 ± 0.1 nM (n_H = 2.3 at 10–90% saturation, r = 0.97) was obtained. The yield of κ-sites solubilized with digitonin was 45%. Similarly high receptor densities in the soluble state were found for [^{3}H]dihydromorphine and for the antagonist [^{3}H]naloxone (table 3).

4. DISCUSSION

Studies at the brain membrane level (to be reported elsewhere) have shown that Mg^{2+} increases the affinity and the capacity of [^{3}H]DADLE binding to the opioid receptor. At 6 mM Mg^{2+} the ligand association rate is relatively slow and requires up to 60 min at 30°C to reach a plateau. These observations led to the pretreatment of membranes with Mg^{2+} which is effective here. Opioid receptors solubilized with digitonin/10 mM Mg^{2+} from such membranes retain their high-affinity binding for the opioid peptides DADLE and dynorphin(1–9) as well as for opiates, when the assay is also in the presence of Mg^{2+}. In particular, the affinities obtained for these ligands are similar to those observed in membrane homogenates under similar conditions. This stable soluble preparation contains, in fact, high

affinity μ, δ, $\varkappa$ and antagonist sites at relatively high densities (table 3).

Of the other detergents tested, only CHAPS-solubilized opioid-receptor sites were able to bind specifically [^{3}H]DADLE and opiates. However, these sites were of lower affinity (not shown) and specific activity. Furthermore, due to high non-specific binding this preparation was not suitable for assaying receptor activity at high ligand concentrations. The B_{max} value for [^{3}H]etorphine and [^{3}H]DADLE obtained in the CHAPS-soluble receptor was 0.18 pmol/mg protein for both: this is 2–3 times higher than reported in [3] for CHAPS-soluble receptor. The low % yields reported here in CHAPS are on the basis of the relatively high densities which we obtain for these ligands [B_{max} 0.63 ([^{3}H]etorphine) and 0.45 pmol/mg protein ([^{3}H]DADLE)] in membrane homogenates under similar conditions. Apart from that factor, we cannot compare any of our yields directly with the values reported for CHAPS in [3], since in that work apparent yields were calculated as the ratio of the soluble to the membrane receptor specific activities (independent of the amount of protein solubilized), whereas ours are the true yields of total activity.

The Hill coefficients obtained for [^{3}H]DADLE or [^{3}H]dynorphin(1–9) suggest strong positive cooperativity. Coefficients of the order of 1.3 have also been reported in [3] for CHAPS medium. In the digitonin medium here, n_H values range from 1.5 (δ and μ) to 2.3 ($\varkappa$). These values can be interpreted in terms of aggregation or disaggregation of opioid receptor subunits on binding the ligand, as also appears to be the case in the membrane-bound receptor (to be reported elsewhere). Hill coefficients of this order have been shown to be generated by such a system in the theoretical models discussed in [12].

The ability of the present preparation to yield soluble receptor of several subtypes in solution will permit investigation of these as individual proteins or subunits.

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